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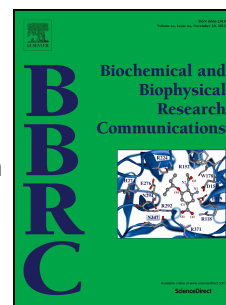
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The effect of surface charge on the thermal stability and ice recrystallization inhibition activity of antifreeze protein III (AFP III)

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Abstract

The aim of this study was to examine the effect of chemical cationization on the structure and function of antifreeze protein III (AFP III) over an extreme temperature range (-40 °C to +90 °C) using far-UV synchrotron radiation circular dichroism (SRCD) and ice recrystallization inhibition (IRI) assays. Chemical cationization was able to produce a modified AFP III with a net cationic charge at physiological pH that had enhanced resistance to denaturation at elevated temperatures, with no immediate negative impact on protein structure at subzero temperatures. Furthermore, cationized AFP III retained an IRI activity similar to that of native AFP III. Consequently, chemical cationization may provide a pathway to the development of more robust antifreeze proteins as supplementary cryoprotectants in the cryopreservation of clinically relevant cells.

Keywords; Antifreeze protein, Synchrotron radiation circular dichroism, ice recrystallization, thermal stability, cryopreservation

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Abbreviations; SRCD, synchrotron radiation circular dichroism. IRI, Ice recrystallization inhibition. AFP III, antifreeze protein III. Myo, equine skeletal muscle met-myoglobin. sfGFP, superfolder green fluorescence protein.

1. Introduction

Cryopreservation is essential for prolonging the longevity of cells, tissues and organs for transplantation.[1] Ice recrystallization during thawing is recognised as a major contributor of cell damage during the cryopreservation process.[2] Therefore, ice recrystallization inhibition (IRI) is key to improving cryopreservation as the demand for the long term storage of clinically relevant cells, tissues and organs increases. In nature, extremophiles have evolved several mechanisms that allow them to thrive in both arctic and antarctic environments. An understanding of these cryotolerant processes began with the isolation and characterisation of antifreeze proteins (AFPs)[3] and (highly conserved) antifreeze (glyco)proteins (AF(G)Ps)[4] in polar fish species, which displayed potent IRI activity.[5,6] Significantly, numerous studies demonstrate the application of AFPs, AF(G)Ps and synthetically derived mimics as supplementary cryoprotectants, enhancing the recovery of various cells and tissues after cryopreservation during thawing.[7,8] However, their application must be tightly controlled due to concentration dependent dynamic ice shaping that heightens damage due to detrimental changes in ice crystal morphology.[9] Within the multitude of protein structures reported, the presence of hydrophobic (proline) amino acid sidechain domains is reported to be of particular importance for the maintenance of ice-protein interactions and IRI activity.[10,11]

The reengineering of proteins *via de novo* gene design, directed evolution or *in silico* approaches is beginning to allow the prediction of subsequent secondary structure and function.[12] An example of protein surface engineering includes the conversion of surface accessible acidic residues into aminated species (supercharging), resulting in an increase in the isoelectric point. Here, the modifications give rise to large positive surface charge densities at physiological pH, which can allow alternative mechanisms for biomolecular conjugation (e.g. polymers) and protein-protein interactions.[13] Moreover, supercationic proteins have exhibited advantageous thermal stability.[14]

Here we examine the antifreeze protein, AFP III, assessing the impact of chemical cationization on secondary structure over an extreme temperature range (-40 °C to +90 °C)

using far-UV synchrotron radiation circular dichroism (SRCD). We compare and contrast these results with the mesophilic model proteins *equine* skeletal muscle met-myoglobin (Myo) and superfolder green fluorescent protein (sfGFP). By quantifying the impact on AFP III functionality with respect to IRI, we demonstrate that cationization increases the high temperature stability of AFP III, but does not reduce IRI capacity, which indicates that changes in surface charge density do not have a critical role in the IRI activity of AFP III.

2. Materials and methods

2.1. Materials

All materials unless otherwise specified were purchased from Sigma-Aldrich Company Ltd (Poole, UK) and were of the highest available purity. Antifreeze Protein III from *Macrozoarces americanus* was purchased from AF Proteins INC (Boston, USA). Superfolder green fluorescent protein[15] (sfGFP) was expressed (E.coli), purified (IMAC) and lyophilized according to standard protocols.

2.2. Surface charge estimation of native proteins

Visualisation of surface charge on native proteins was calculated from existing PDB files via their conversion into PQR files and analysis with the Adaptive Poisson-Boltzmann Solver software package to determine biomolecular solvation and electrostatics prior to visualisation in visual molecular dynamics.[16] Theoretical net charge and isoelectric point values were calculated using the online tool "Protein Calculator v3.4".[17]

2.3. Cationization of native proteins

Protein Cationization followed a similar procedure as previously reported.[18] In brief 1 mg.mL⁻¹ of protein in 20 mM HEPES was added to (pH7 adjusted) N,N'-dimethyl-1,3-propanediamine equivalent to 150-fold the number of cationizable sites along with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride equivalent to 34-fold the number of cationizable sites. This mixture was adjusted to pH 6 and stirred overnight at 25 °C. Samples were then filtered (0.22 µm) to remove aggregates and dialysed (3.5 kDa MWCO) before lyophilisation with cationization evaluated by matrix assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF MS) and zeta potentiometry.

2.4. Measurement of IRI activity

Ice recrystallization inhibition (IRI) measurements (mean largest grain size) were performed using a "splat" assay as previously reported[19], but all tests were conducted in

solutions of 20 mM HEPES (pH7). A complimentary analysis of IRI activity was performed using MATLAB (The MathWorks. INC., MA, USA) allowing estimation of the diameter and distribution of the ice crystals. In brief, the image processing algorithmic analysis for the evaluation of the difference in granularity was carried out in a MATLAB environment where images were converted into a binary format, via thresholding, where pixels with value 0 correspond to the background and with value 1 to the object of interest. Thresholding was adapted for each image so the majority of the information was maintained for analysis. The binary images generated were then labelled through 8-connected component labelling through the `bwlabel()` MATLAB function, which enables the detection of connected regions in binary images, in our case grains. This operation enabled pixels with values of 1 (since this is a binary image) to be 'connected' together to form a grain (through a unique label) while pixels of value of 0 (background pixels) were excluded. Using the information of connected labelling, the function `regionprops()` was used to estimate the diameter of each component based on an equivalent circle estimation. This information was then plotted as a histogram to determine the different grain sizes apparent in each image and their frequency.

2.5. Assessment of protein thermal stability

Thermal stability of native and cationized proteins was performed using a Cary 60 UV-visible spectrometer (Agilent Technologies LDA UK Ltd., UK) equipped with a Quantum Northwest TC1 temperature controller (Quantum Northwest Inc., USA). 0.5 mg.mL⁻¹ samples (pathlength 1 cm) were measured continuously at a rate of 0.5 °C.min⁻¹ for sfGFP and 1 °C.min⁻¹ for AFP III and Myoglobin from +25 °C to +85 °C at 300 nm.

2.6. SRCD of proteins

Synchrotron radiation circular dichroism (SRCD) was conducted on beamline B23 at the Diamond Light Source synchrotron facility (Didcot, UK). Samples assessing high

temperature denaturation and refolding were prepared in 10 mM sodium phosphate (pH 7.3) at 0.6 mg.mL⁻¹ with a 0.02 cm pathlength between 260 nm – 190 nm at 1 nm intervals and a 2 second integration time at temperatures ranging from +20 to +90 °C (and vice versa) at 5 °C intervals. Samples were heated (or cooled) at 1 °C.min⁻¹ and held at the target temperature for 5 minutes prior to measurement. Samples assessing the effect of ethylene glycol were prepared in 10 mM sodium phosphate (pH 7.3) with the appropriate amount of ethylene glycol (0 % -100 % (v/v) at 20 % (v/v) intervals) with 0.6 mg.mL⁻¹ protein at a 0.02 cm pathlength between 260 nm – 190 nm at 1 nm intervals and a 4 second integration time at 25 °C. Samples assessing low temperature were prepared in 10 mM sodium phosphate (pH 7.3) with 60 % (v/v) ethylene glycol (freezing point -45 °C) with 0.6 mg.mL⁻¹ protein at a 0.02 cm pathlength between 260 nm – 190 nm at 1 nm intervals and a 1 second integration time at temperatures ranging from +20 to -40 °C, cooling at 1 °C.min⁻¹ and held at the target temperature for 5 minutes prior to measurement.

3. Results and Discussion

Native AFP III (**Fig. 1A**), which comprises a mixture of isoforms[20], was chemically cationized using a small molecule diamine. This was achieved using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride mediated nucleophilic addition of N,N'-dimethyl-1,3-propanediamine to glutamic acid and aspartic acid residues. Cationization of native AFP III resulted in a 280 Da increase in mass, as adjudged by MALDI-TOF MS (**Fig. 1B**). This corresponded to the modification of 3.3 residues per protein on average (34.5 % efficiency) though a distribution of cationized species as shown by a broadening of the cationized MALDI-TOF MS peak are formed. This equates to a theoretical isoelectric point shift from pH 6.2 to \approx 11.2 and theoretical predicted net charge of +5.9 at pH 7. Changes in surface charge were supported by zeta potentiometry, which showed a shift in the zeta potential maxima from -3.0 mV to +1.3 mV at pH 7 (**Fig. 1C**).

[INSERT FIGURE 1]

To investigate the impact of cationization on protein secondary structure, far-UV SRCD experiments were performed. At 20 °C (**Fig. 2A**), both native and cationized AFP III exhibited spectra that were consistent with a high degree of disordered structure[21], although there was a reduction in the magnitude of the minimum at 196 nm with cationized AFP III. Accordingly, an (CDSSTR)[22] analysis of the SRCD spectra was used to estimate the effect of cationization on secondary structure content (**Fig. 2B**), which showed a slight decrease in the β -sheet structure (41% to 39%), accompanied by a concomitant increase in disordered content (31% to 34%). These differences are likely the result of changes in the distribution of intramolecular salt bridges and hydrogen bonding after cationization.[23,24]

To test the impact of cationization on thermal stability, SRCD measurements were performed over a temperature range of +20 – +90 °C and changes in the AFP III secondary structure were estimated by monitoring the change in the mean residue ellipticity ($[\theta]$) at 196 nm. Native and cationized (68% efficiency; **Supp. Fig. 1A-B**) equine skeletal muscle met-

myoglobin were used as controls, and due to their predominantly α -helical structure[25], temperature dependent changes were inferred using the mean residue ellipticity ($[\theta]$) at 222 nm. For native AFP III, heating to +90 °C resulted in a large reduction (ca. 90%) in the mean residue ellipticity ($[\theta]$), which was indicative of thermal denaturation (**Fig. 2C**). This was accompanied by a gradual shift in the minima from 197 nm to 203 nm and a reduction in signal intensity at 222 nm. Shifts in the CD minima have been reported for collagen II (under comparable thermal denaturation conditions) that displays similar disordered structure[26], and for AFP III when subjected to chemical denaturation conditions using sodium dodecyl sulphate.[21] Conversely, only a 47% reduction was observed for cationized AFP III (**Fig. 2D**), with a less pronounced shift in the minima and concurrent reduction in signal intensity. This protective trend was also observed for native and cationized myoglobin from +20 – +90 °C, *i.e.*, for native myoglobin (**Fig. 2E**) approximately 50% of the SRCD signal was retained, whereas for cationized myoglobin, there was no significant loss of signal (**Fig. 2F**). The degree of refolding on cooling to +20 °C from +90 °C varied between the native and cationized proteins. Here, both native AFP III and native Myo showed minimal recovery of secondary structure after cooling (**Fig. 2G-H**). Conversely, cationized AFP III showed greater recovery of the SRCD signal, while cationized Myo also maintained its folded configuration.

[INSERT FIGURE 2]

The lack of reversibility on cooling can be rationalised by thermal denaturation leading to irreversible aggregation of the native proteins. After cationization, however, the increase in the repulsive electrostatic protein-protein interactions may have inhibited protein aggregation. Indeed, a *de novo* supercharged GFP +36 variant of superfolder GFP (sfGFP) has been reported to have improved thermal stability with respect to aggregation resistance.[14] Accordingly, we investigated whether this also applied to chemically cationized proteins including chemical cationized sfGFP (80% efficiency; **Supp. Fig. 1C-D**).

Here, turbidity measurements were performed by monitoring the change in absorbance at 300 nm from +25 – +85 °C at either 0.5 or 1 °C.min⁻¹ (**Supp. Fig. 2A-C**). Significantly each native protein exhibited the onset of aggregation at a lower temperature when compared with their chemically cationized equivalent, which supports the previous finding that supercharging improves resistance to thermal aggregation.

To assess cold denaturation, far-UV SRCD studies (including sfGFP) were conducted from +20 – -40 °C, which is above and below the physiological temperature (\approx -2 °C) of *Macrozoarces americanus*.^[20] Measurements necessitated the addition of an organic solvent as an antifreeze to inhibit scattering from ice crystals. Accordingly, the effect of the addition of ethylene glycol at various concentrations was monitored for each protein (**Supp. Fig. 3**). For native AFP III and native sfGFP, the secondary structure distribution was relatively constant up to 80 % (v/v), although a decrease in the disordered structure and β -sheet content were observed at 100 % (v/v) ethylene glycol for native AFP III and native sfGFP, respectively. Conversely, native Myo showed a large α -helical to β -sheet shift in the secondary structure distribution at only 20 % (v/v). However ethylene glycol has been reported to induce shifts in secondary structure typically towards α -helical configurations with other proteins,^[27] though in the presence of the organic solvent, trifluoroethanol, such shifts appear to be protein specific.^[28] Although the secondary structure distribution of native AFP III did not appear to vary significantly at the lower concentrations of ethylene glycol (20 % – 80 % (v/v)), the minimum at 197-200 nm shifted towards 203-208 nm and a band at 222 nm became apparent. These changes are consistent with other intrinsically disordered proteins in solutions containing 0-65 % (v/v) trifluoroethanol.^[29,30]

Consequently, far-UV SRCD measurements were performed from +20 – -40 °C in the presence of 60 % (v/v) ethylene glycol (**Fig. 3A-B**), though temperatures relevant to long-term cryopreservation^[1] would have necessitated concentrations of organic solvents incompatible with SRCD.^[31] Both native and cationized AFP III exhibited distinctive spectral features at approximately 198 nm and 220 nm, that varied as the temperature was decreased from +20 to -40 °C. The slight increase in intensity at 198 nm and reduction at

220 nm is consistent with the stabilisation of a polyproline type II structure as reported with an alanine-based peptide[32] and human papilloma virus 16-E7 protein[30], which are both intrinsically disordered. However, changes in the spectra upon cooling were not reflected in secondary structure composition of native and cationized AFP III after deconvolution (**Fig. 3C-D**). This was also the case for native and cationized myoglobin and sfGFP (**Supp. Fig. 4**), which gave rise to similar CDSSTR profiles at both +20 and -40 °C. The absence of quantitative structural changes describing cold denaturation of the proteins may have been a result of the low sensitivity of the CDSSTR approach, although cryoprotection of the protein structure from the ethylene glycol could not be ruled out.

[INSERT FIGURE 3]

The IRI activity of AFP III is key for its potential as a supplementary cryoprotectant. Therefore, to probe the impact of chemical cationization, the IRI activity of cationized AFP III at 100 $\mu\text{g.mL}^{-1}$ was assessed using a modified splat assay[19] by measuring ice crystal size 30 minutes post nucleation at -6 °C (promotes rapid recrystallization). This was compared to native AFP III, which at 100 $\mu\text{g.mL}^{-1}$ has been reported to possess a strong IRI activity[33] with the onset of detectable IRI activity reported at concentrations as low as approximately 5 $\mu\text{g.mL}^{-1}$ [34]. Furthermore, 9 kDa poly(vinyl alcohol) (PVA) at 5 mg.mL^{-1} was used as a non-protein IRI positive control[35]. Each micrograph highlighted potent IRI activity compared to a 20mM HEPES negative control ($126 \mu\text{m} \pm 13 \mu\text{m}$) (**Fig. 4A**) with an approximate mean ($n=10$) largest grain size (MLGS) of $30.2\% \pm 4.8\%$, $26.6\% \pm 3.9\%$ and $34.7\% \pm 5.4\%$ for native AFP III, cationized AFP III and PVA, respectively (**Fig. 4B-D**). Using a complimentary image analysis algorithm, ice crystals in the native AFP III, cationized AFP III and PVA micrographs were identified, and their size distribution plotted (**Fig. 4E**). Consequently, the mean and standard deviation of the 500 largest crystals (as the largest crystals incite cryodamage) were calculated as $26.0 \pm 23.8 \mu\text{m}$, $27.7 \pm 10.6 \mu\text{m}$ and $29.2 \pm 13.1 \mu\text{m}$ for native AFP III, cationized AFP III and PVA respectively. These values emphasize minimal

ice recrystallization as they are not significantly different to 100 $\mu\text{g.mL}^{-1}$ native AFP III imaged immediately after nucleation which had a MLGS of $29.1\% \pm 7.7\%$ (**Supp. Fig 5**). The insignificant differences between native and cationized AFP III indicate that alteration of surface charge does not impact significantly on the IRI activity of AFP III at a concentration that aids the cryopreservation of red blood cells.[7]

[INSERT FIGURE 4]

These outcomes are consistent with mutagenesis and computational studies of AFP III, which reported that conserved proline residues contribute to observed antifreeze activity (thermal hysteresis) and protein folding *in vivo* [36,37] and that ice binding sites in AFPs are predominantly hydrophobic.[10,11,38,39] Our findings are further supported by a recombinant AFP III variant which differed by the inclusion of 2 additional (cationic) lysine residues and 1 additional methionine residue which not only displayed a slightly enhanced IRI activity but also an elevated resistance to thermal degradation.[40] The same study also showed circular dichroism spectroscopy measurements at 0 °C that displayed no significant differences in spectra. Nonetheless qualitative effects on our SRCD spectra were apparent between native AFP III and cationized AFP III which could be accounted by the greater number of modifications and correspondingly larger shift in charge having a more significant impact but as aforementioned chemical cationization did not result in a reduction of IRI activity at 100 $\mu\text{g.mL}^{-1}$. As the cationization process is dependent on the reactivity of the nucleophile, but importantly, the solvent accessibility of the target amino acid carboxylate group, it is conceivable that the cationization process could be tuned (e.g. pH, temperature, reaction time) to maximise thermal stability.

In conclusion, we have demonstrated that cationization can be used to stabilise protein structure with respect to thermal aggregation without reductions in IRI activity. At subzero temperatures, there was little evidence of cold denaturation for any of the proteins, although stabilising effects from the presence of ethylene glycol cannot be ruled out.

Accordingly, chemical cationization may provide a pathway for the development of new robust protein-based cryoprotectants.

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Figures

Fig. 1 | Charge distribution and cationization of AFP III. **A.** Solvent-exposed surface of native AFP III (PDB;1KDF) structure displaying the distribution of positive (blue) and negative (red) surface charges. **B.** MALDI-TOF MS of native (red) and cationized AFP III (blue) at pH7 and **C.** Zeta potential of native (red) and cationized AFP III (blue) at pH7.

Fig. 2 | Thermal stability of native and cationized proteins. **A.** SRCD spectra of native (solid line) and cationized AFP III (dotted line) at 20 °C. **B.** Secondary structure distribution (CDSSTR) of native and cationized AFP III. Red, blue, green and black represent α -helical, β -sheet, turns and disordered content (%) respectively. SRCD spectra of samples heated from 20 °C to 90 °C (blue to red) at 5 °C intervals for **C.** native AFP III **D.** cationized AFP III **E.** native myoglobin and **F.** cationized myoglobin. Inserts represent changes in relative signal intensity at 196 nm (AFP III) or 222 nm (myoglobin). SRCD spectra at 20 °C before (blue) and after (green) heating to 90 °C (red) for **G.** native AFP III (solid lines) and cationized AFP III (dotted lines) and **H.** native myoglobin (solid lines) and cationized myoglobin (dotted lines).

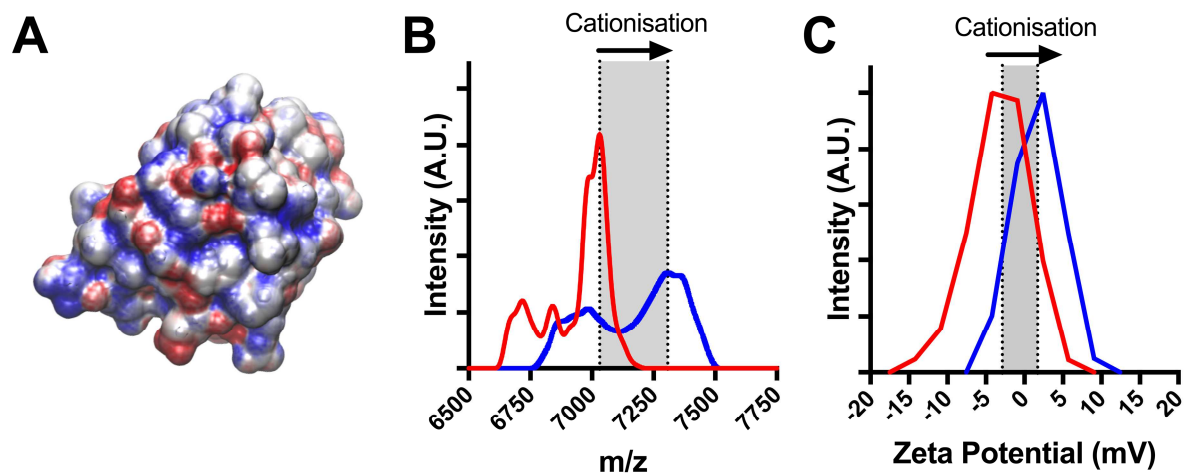
Fig. 3 | Cold stability of AFP III. SRCD spectra of **A.** native and **B.** cationized AFP III at temperatures between +20 °C and -40 °C in 60 % (v/v) ethylene glycol. Secondary structure (CDSSTR) composition of **C.** native and **D.** cationized AFP III at +20 °C and -40 °C. Red, blue, green and black represent α -helical, β -sheet, turns and disordered content respectively.

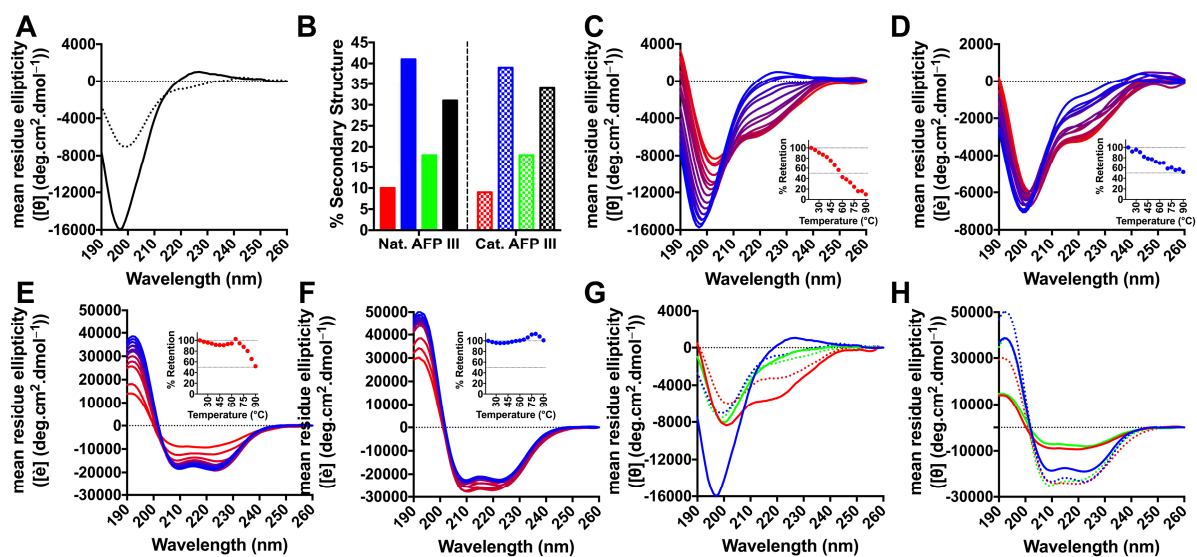
Fig. 4 | Ice recrystallization inhibition properties of AFP III. **A.** 20mM HEPES and ice recrystallization inhibition of 20mM HEPES supplemented with **B.** 100 $\mu\text{g.mL}^{-1}$ native AFP III, **C.** 100 $\mu\text{g.mL}^{-1}$ cationized AFP III and **D.** 5 mg.mL^{-1} 9 kDa PVA. Scale bar represents 200 μm . **E.** Frequency distribution of ice crystal diameter distribution ($10^x \mu\text{m}$) as adjudged by granularity analysis of native AFP III (blue), cationized AFP III (red) and 9 kDa PVA (green).

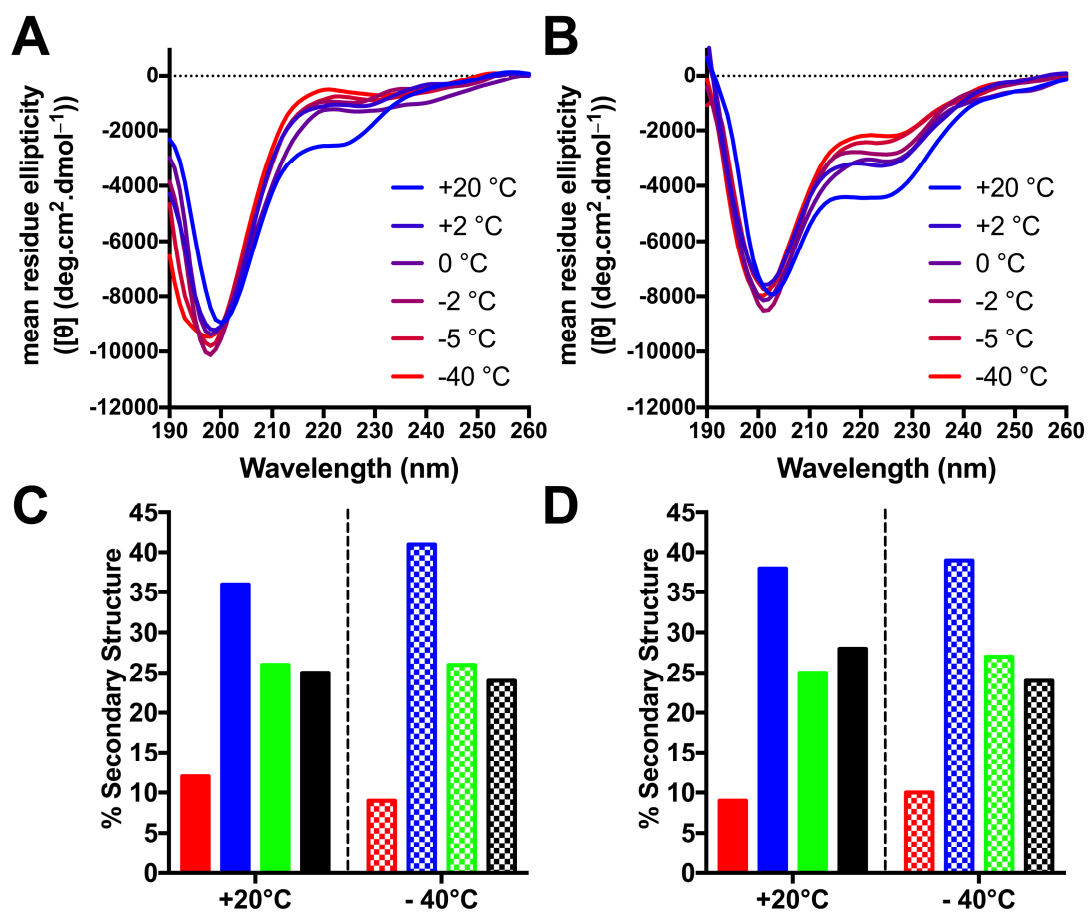
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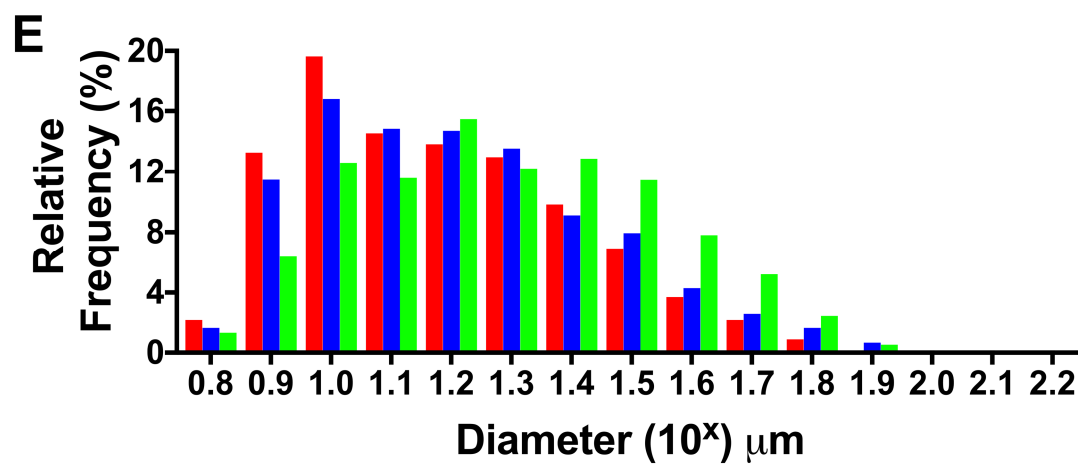
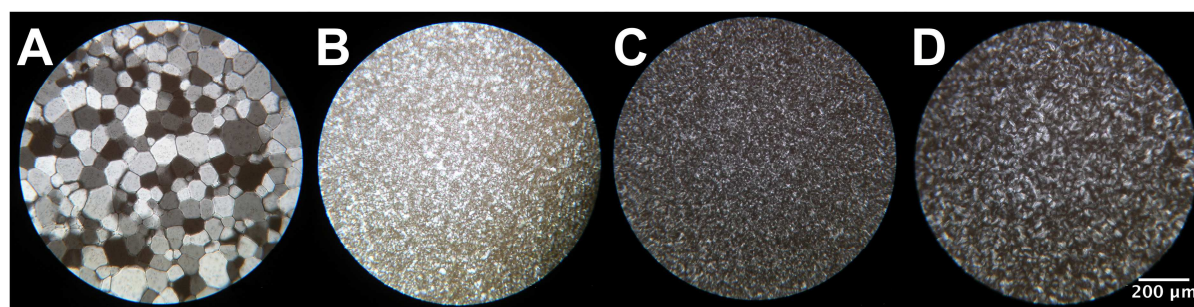
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The effect of surface charge on the thermal stability and ice recrystallization inhibition activity of antifreeze protein III (AFP III)

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Highlights

- Chemical cationization improves thermal stability of antifreeze protein III.
- Cationic antifreeze protein III retains ice recrystallization inhibition activity.
- Synchrotron radiation circular dichroism investigation of antifreeze protein III.